Leukemia Research 37 (2013) 631–636

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres

Lipoprotein lipase in chronic lymphocytic leukaemia – Strong biomarker with lack of functional significance

Edit Porpaczy[a,b], Stefanie Tauber[c], Martin Bilban[b,d], Gerhard Kostner[e], Michaela Gruber[a,b], Sandra Eder[f], Daniel Heintel[g], Trang Le[a,b], Karin Fleiss[a,b], Cathrin Skrabs[a,b], Medhat Shehata[a,b], Ulrich Jäger[a,b,h], Katrina Vanura[a,b,h,*]

[a] Department of Medicine I, Division of Hematology and Hemostaseology, Medical University of Vienna, Austria
[b] Comprehensive Cancer Center, Medical University of Vienna, Austria
[c] Center for Integrative Bioinformatics Vienna (CIBIV) Max F Perutz Laboratories, Medical University of Vienna, University of Vienna, Austria
[d] Department of Clinical Chemistry and Laboratory Medicine, Medical University of Vienna, Austria
[e] Department of Molecular Biology and Biochemistry, University of Graz, Austria
[f] Department of Clinical Pharmacology, Medical University of Vienna, Austria
[g] Medical Department I, Center for Oncology and Hematology, Wilhelminenspital, Vienna, Austria
[h] German CLL Study Group, Köln, Germany

ARTICLE INFO

Article history:
Received 29 October 2012
Received in revised form 5 February 2013
Accepted 9 February 2013
Available online 7 March 2013

Keywords:
CLL
LPL
Functional role
Knock down

ABSTRACT

In chronic lymphocytic leukaemia (CLL), lipoprotein lipase (LPL) mRNA overexpression is an established poor prognostic marker, its function, however, is poorly understood. Measuring extracellular LPL enzymatic activity and protein, we found no difference between levels in CLL patients and those of controls, both before and after heparin treatment *in vivo* and *in vitro*. Investigating LPL knock down effects, we determined five potential downstream targets, of which one gene, STXBP3, reportedly is involved in fatty acid metabolism.

While possibly reflecting an epigenetic switch towards an incorrect transcriptional program, LPL overexpression by itself does not appear to significantly influence CLL cell survival.

1. Introduction

Chronic lymphocytic leukaemia (CLL) is the most frequent leukaemia in Western countries. Median age at diagnosis is 64–70 years, survival times differ considerably between patients and range from normal life expectancy (>20 years) to a few years in high-risk groups [1,2]. Prognostic factors include clinical staging systems (Rai and Binet), cytogenetics, serum markers, expression of surface molecules, and genetic markers, particularly the mutational status of immunoglobulin heavy chain variable region genes (IGHV) [1,3–6]. Several surrogate markers for IGHV mutational status have been identified in recent years, ZAP-70 protein and lipoprotein lipase (LPL) mRNA expression being the most reliable ones [7–11]. LPL predicts overall and treatment free survival [12–18]. LPL mRNA expression alone or integrated into scoring systems have established its prognostic value in CLL [19–22].

Given the central role of LPL in lipid metabolism it is attractive to speculate on the impact of its overexpression on the survival of CLL cells. However, data on the functional role of LPL in CLL are very rare and in part conflicting. Our previous data suggest that LPL protein is also found on the cell surface of CLL cells but that mRNA overexpression is mainly associated with an increase of *intracellular* LPL protein [23]. We therefore hypothesized that CLL cells can synthesize LPL protein that in part is secreted and bound to the cell membrane [12]. A correlation of LPL mRNA with protein expression in CLL was also observed by Mansouri and colleagues, who suggested that the majority of protein might be catalytically inactive [24]. On the other hand, Wendtner and colleagues suggested that lipid metabolism is causally related to the survival of CLL cells based on the induction of apoptosis by tetrahydrolipostatin (Orlistat) or erufosine, a synthetic phospholipid analogue with antineoplastic activity, treatment *ex vivo* [25,26]. *In vivo*, decreased high density lipoprotein cholesterol (HDL-C) levels were observed in the plasma of CLL and ALL patients compared to healthy individuals [27]. Nevertheless, so far there are no published data, which prove a distinct and specific functional role of LPL in CLL.

LPL function is best characterized in endothelial cells [28,29]. LPL is produced in parenchymal cells and transported to

* Corresponding author at: Medical University of Vienna, Department of Medicine I, Division of Hematology and Hemostaseology, Waehringer Guertel 18-20 1090 Vienna, Austria. Tel.: +43 1 40400 73781; fax: +43 1 40400 73595.
E-mail address: katrina.vanura@meduniwien.ac.at (K. Vanura).

0145-2126 © 2013 Elsevier Ltd. Open access under CC BY-NC-ND license.
http://dx.doi.org/10.1016/j.leukres.2013.02.008

endothelial cells that bind LPL by heparan sulfate–proteoglycan (HSPG) on the luminal cell surface [28]. Intravenous injection of heparin competes for these HSPG binding sites and liberates LPL into circulation, leading to an increase in LPL plasma protein concentration and enzymatic activity [28]. This so called post heparin lipolytic activity (PHLA) is an established diagnostic test for lipid metabolism disorders.

In the present study we assessed LPL protein concentration and enzyme activity in serum and plasma of CLL patients with high or low LPL mRNA expression before and after heparin treatment. These *in vivo* experiments were complemented by *in vitro* treatment of CLL primary cells with heparin and purified LPL protein. LPL knockdown in CLL cells by siRNA was performed to study changes in functional behaviour of CLL cells and gene expression.

2. Patients, materials and methods

2.1. Patients

A total number of 114 fully characterized, untreated chronic lymphocytic leukaemia patients diagnosed at the Division of Haematology at the Medical University of Vienna (Austria) were retrospectively screened for LPL mRNA expression and survival curves were calculated. In this cohort, we confirmed the prognostic power of LPL mRNA expression regarding OS and TFS (Supplementary data and Supplementary Fig. 1). Forty-two patients, for which serum and plasma samples were available, were studied with respect to LPL protein levels in serum and LPL enzymatic activity in plasma. Of these 42 patients, thirteen patients were selected to study LPL release after heparin injection. Seventeen of these patients were selected for LPL knock down experiments. Selection criteria were (1) availability of material needed for knock down and (2) high LPL mRNA expression at sample date, with the goal to maximize mRNA expression reduction through knock down. All patients were untreated. Basic clinical information for the study cohorts is listed in Supplementary Table 1. Three healthy individuals (HIs) and 14 non-CLL patients (lymphoma patients in complete remission) served as controls. All participants gave informed consent according to the Declaration of Helsinki, approval of the institutional Ethics Committee was obtained for this study (Ethics Committee number: 035/2007). Serum, plasma and peripheral blood cells were collected. Peripheral blood mononuclear cells (PBMC) were isolated using standardized Ficoll–Hypaque gradient centrifugation (Seromed, Berlin, Germany).

2.2. Cell culture

Primary CLL PBMC, cells of the cell lines HeLa (cervix carcinoma), Hep3B (hepatocellular carcinoma), CCL228 (colon carcinoma), THP1 (monocytic leukaemia) and RPMI8226 (plasma cells of multiple myeloma) were cultured at 37 °C and 5% CO_2 in GIBCO RPMI 1640 (Life Technologies, Carlsbad, CA, USA) containing 10% foetal calf serum (FCS Gold), 1% penicillin/streptomycin (PS) and 1% L-glutamine (all PAA Laboratories, Linz, Austria).

2.3. Heparin provocation test in vivo

To investigate *in vivo* mobilization of LPL in CLL patients (PHLA), a heparin release study on a cohort of 13 fully characterized CLL patients, was performed. This cohort represents consecutive CLL patients, which consented in writing to participate in this part of the study. Patients received 50 U/kg body weight of standard heparin intravenously (Heparin Immuno, Baxter BioScience, Vienna, Austria). Serum and blood samples were collected before, 10 and 20 min after heparin injection. LPL protein levels in serum, LPL enzymatic activity in plasma, LPL mRNA expression in PBMC and viability of CD3, CD14 and CD19 positive cells were investigated in all samples. For ethical reasons, published values of healthy individuals before and after heparin administration were used as reference.

2.4. Heparin provocation test and LPL treatment in vitro

LPL mobilization *in vitro* was assessed in three (one mutated and two unmutated) CLL samples. Immediately after Ficoll isolation, PBMC were washed twice with phosphate-buffered saline (PBS), counted and 5×10^6 cells were cultured in 5 ml serum-free GIBCO RPMI 1640 containing 1% PS for 2 h at 37 °C and 5% CO_2. Then cells were treated with 20 U standard heparin. After 20 min of incubation, supernatant was harvested and stored at −20 °C for LPL protein and enzymatic activity measurements. Cells were instantly stained and apoptosis was analyzed by flow cytometry. THP1 cells cultured and treated under the same conditions were used as positive control. To evaluate the effect of external LPL on cell viability, cells of the 3 CLL patients were cultured and treated for 24, 48 and 72 h with 10, 100, 1000, and 10,000 ng/ml of purified LPL protein (Sigma, St. Louis, MO, USA). Apoptosis was investigated by flow cytometry.

2.5. LPL protein levels and enzymatic activity

Pre-heparin serum and plasma of 42 CLL, 14 non-CLL patients (lymphoma patients in complete remission) and 3 HIs, as well as pre- and postheparin serum and plasma from 13 CLL patients selected for heparin provocation tests were collected and stored at −20 °C until further analysis. Enzyme-linked immunosorbent assay (ELISA) was performed using the LPL EIA kit (ALPCO diagnostics™, Salem, NH, USA) according to the manufacturer's instructions. Before use, serum was diluted 1:30. Linearity of the assay system was observed from 9 to 500 ng/ml, the coefficient of variation (Cv) was less than 10% (within-run). LPL enzymatic activity assays were carried out at the Department of Molecular Biology and Biochemistry at the University of Graz as described elsewhere [30].

2.6. LPL mRNA expression

RNA and cDNA preparation and real-time PCR were carried out as described previously (LPL TaqMan assay-on-demand: Cat. No. Hs00173425_m12, Life Technologies, Carlsbad, CA, USA) [12].

2.7. Apoptosis and viability

To determine cell phenotype, PBMCs were washed with PBS and re-suspended in 0.3% BSA (bovine serum albumin) and 0.1% Na-azide (both Sigma, St. Louis, MO, USA). Nonspecific binding was blocked by 20% human AB serum (Cat. No. C11-021, PAA Laboratories, Linz, Austria) in PBS/BSA. Cells were stained with mouse anti-human CD3, CD19, CD14 – PE (BD Biosciences, San Diego, CA, USA) using the recommendations of the manufacturer. Cells were incubated for 30 min on ice, washed, re-suspended in 0.2 ml PBS/BSA and analyzed on a Becton Dickinson FACScan. For each analysis 10,000 events were acquired and analyzed using the CellQuestPro software.

For apoptosis detection, the Annexin-V detection kit (Bender MedSystems, Vienna, Austria) was used according to the manual and analyzed on the BD FACScan. The rate of cellular apoptosis was calculated as the percentage of Annexin positive cells.

Viability was detected using the Cell-Titre Blue Assay according to the instructions of the manufacturer (Promega, Madison, WI, USA).

2.8. Transfection of primary CLL cells

Stealth Select 3 RNAi™ set designed to silence human LPL, Stealth RNAi™ scrambled siRNA negative controls for high and low GC-content, and Block-iT™ positive control Fluorescent Oligo for lipid transfection were acquired from Life Technologies (Carlsbad, CA, USA). All three siRNAs targeting LPL were tested and the most effective was used for subsequent experiments. PBMC of 10 CLL patients with high LPL mRNA expression were selected for knock down experiments. Cells were thawed, counted and 10×10^6 cells per well were plated in 6-well cell culture plates using 2 ml phenol red free GIBCO RPMI 1640 (Life Technologies, Carlsbad, CA, USA) containing 10% FBS, 1% L-glutamine, and 1 ng/ml TPA (phorbol-12-myristate-13-acetate, Sigma Aldrich, St. Louis, MO, USA). Cells were then incubated for 1 h at 37 °C. RNAi duplexes and 15 μl Lipofectamine™ 2000 Reagent (Life Technologies, Carlsbad, CA, USA) were diluted in 500 μl Opti-MEM (Life Technologies, Carlsbad, CA, USA) and incubated for 20 min at room temperature according to the manufacturer's instructions. Five hundred microlitres of Stealth™ RNAi-Lipofectamin complexes were added to each well, final siRNA concentration was 60 pmol/ml. Each transfection was performed in duplicate. After 24 h, 1 ml of fresh medium was added to each well. After 48 h, cells were harvested, washed with ice-cold PBS and RNA was prepared using TRIzol. Transfection efficiency and effect on cell viability were monitored by flow cytometry. LPL mRNA expression was measured in all samples (negative control, mock transfection, transfection with scrambled siRNA and transfection with siRNA specific against LPL). The ratio in percent of LPL mRNA expression after transfection with scrambled and LPL specific siRNA, respectively, represents knock down efficiency. In addition, analyses of co-regulated genes were carried out by gene-expression profiling as described below.

2.9. Transfection of cell lines

One day before transfection, cells were seeded in 2 ml culture medium without antibiotics. At 70–80% confluence, cells were transfected. HeLa, Hep3B and CCL228 cells were transfected using Lipofectamine™ 2000 following the cell specific transfection protocols of the manufacturer. RPMI8226 and THP1 cells were transfected using Lipofectamine™ RNAiMAX according to the manufacturer's instructions for THP1 cells. This protocol was modified in one particular aspect: similar to CLL cells, THP1 cells were treated with 1 ng/ml TPA 1 h prior to transfection.

2.10. Microarray analysis

To determine genes up or down regulated after LPL knock down, RNA from paired samples (transfected with scrambled siRNA *vs.* LPL specific siRNA from the same time point) was extracted as described above, purified using the RNeasy Minelute kit (QIAGEN Inc., Valencia, CA, USA). Total RNA (200 ng) was then used for

GeneChip analysis. Preparation of terminal-labelled cDNA, hybridization to genome-wide human Gene Level 1.0 ST GeneChips (Affymetrix, Santa Clara, CA, USA) and scanning of the arrays were carried out according to manufacturer's protocols (https://www.affymetrix.com).

2.11. Bioinformatic analysis

The R Bioconductor Suite (http://www.R-project.org/Bioconductor) has been used for the analysis of the microarray data. RMA was used for normalization [31]. Hierarchical clustering was performed using Pearson's correlation coefficient as distance measure and ward's optimization criteria to cluster genes and samples, respectively. Gene expression data are available at the GEO database under the accession number GSE44247.

3. Results

3.1. LPL protein levels and activity in the serum or plasma of CLL patients

LPL concentrations were measured in the serum of CLL patients with various LPL mRNA levels at baseline and after administration of intravenous heparin using standard methods. No differences in pre-heparin LPL serum protein levels or enzymatic activity were observed when comparing 42 CLL *vs.* 14 non-CLL patients *vs.* 3 healthy individuals (Fig. 1A). Prognostic subgroups within CLL patients (M *vs.* UM, high LPL mRNA *vs.* low LPL mRNA) showed no significant differences (Fig. 1B). LPL serum protein concentration and plasma activity of CLL patients increased after heparin injection *in vivo* ($N = 13$; patient characteristics listed in Supplementary Table 2). Measured levels were in the range of those expected in healthy subjects reported in the literature (Table 1) [30,32–34]. Importantly, no significant difference was observed between patients with high or low LPL mRNA expression in their CLL cells collected at the same time (Table 1).

In line with this observation, no LPL mobilization from CLL cells could be observed after heparin treatment *in vitro*. Cell viability remained unaffected both *in vivo* and *in vitro* after heparin treatment and *in vitro* after incubation with LPL protein (data not shown).

3.2. LPL knock down experiments

In order to determine the functional impact of LPL expression we performed siRNA knock down in primary CLL PBMC and control cell lines, all expressing high LPL mRNA levels (HeLa (expression level 241.77), Hep3B (548.74), CCL228 (74.87), THP1 (51.67 before and 157.31 after stimulation with TPA), and in RPMI8226 cells (121.30). Tumour load and knock down efficiencies are summarized in Supplementary Table 3. Highest knock down (up to 90%) could be reached in adherent cell lines followed by suspension cell lines and CLL primary cells. The rate of apoptosis in all cell lines (even in those with the highest knock down efficiency) and CLL samples was not different in samples transfected with LPL targeting or scrambled siRNA (Supplementary Fig. 2). Comparing a time series up to 72 h, we observed an optimal knock down to apoptosis ratio at 48 h (data not shown).

Due to knock down heterogeneity among CLL samples, only experiments showing an LPL knock down of at least 25% after 48 h (10 of 17) were selected for microarray analysis (median knock down efficiency: 33%; range: 25–54%; Supplementary Table 3). Microarray analysis was also performed in the five cell lines listed above. In unsupervised clustering gene expression profiles after knock down in THP1 cells were most similar to CLL. Among CLL samples, two main clusters (three *vs.* seven samples) with similar gene expression profile after knock down were identified. No clinical differences were found between patients belonging to either cluster (data not shown).

Correlating gene signal intensity and knock down strength, fold changes were calculated for the entire study cohort. Considering a maximum LPL knock down efficiency of 54% in primary CLL cells, we selected the following criteria to achieve the most reliable output regarding specific knock down related changes in gene expression: a cut-off of at least 20% deregulated expression in at least 7 of the 10 primary samples. The resulting genes were then ranked according to the number of CLL samples with similar de-regulation. In total, 33 genes were down-, and 3 genes were up regulated in at least 7 of 10 samples after LPL knock down (Supplementary Fig. 3). Applying a similar restriction to the cell lines, 20% deregulation in at least three of the five cell lines, no overlap in deregulated genes were found between cell lines and CLL samples. Even comparing single cell lines with the CLL cohort, no overlapping genes (at 20% deregulated) could be observed.

Six genes downregulated in 8 of 10 CLL samples were selected for validation with additional samples. Again, 25% LPL knock down efficiency was set as cut off. After this second round of transfections ($N = 7$) and measuring gene expression of the selected genes by real time PCR, the following 5 genes showed significant downregulation together with LPL: LMAN1 (lectin, mannose-binding 1), STXBP3 (syntaxin-binding protein 3), LARP7 (La ribonucleoprotein domain family, member 7), RANBP2 (RAN-binding protein 2), SSB (Sjogren syndrome antigen B) (Table 2). Of these, STXBP3 might be a downstream target of LPL since parallel regulation has been described in conjunction with fatty acid induced insulin resistance of skeletal muscle [35]. Our data show that LPL knock down is associated with specific expressional changes in CLL cells but not necessarily with changes in cell survival at least in short term experiments.

4. Discussion

Reports are accumulating, that fatty acid metabolism is altered in human malignancies [36–38]. LPL, a key enzyme in lipid metabolism, is known to hydrolyze circulating triglyceride-rich lipoproteins. Absence or decreased LPL activity causes an increase of plasma triglycerides (TG) coinciding with reduced HDL-C levels [28,39]. Accordingly, decreased HDL-C plasma levels were reported in patients suffering from lymphoid leukaemia [27]. Heparin injection influences LPL activity, by binding and displacing LPL from HSPG binding sites. Only active as non-covalent homodimer, dimeric LPL appears to have much higher affinity for heparin and HSPG compared to its monomeric form [28].

Here we provide novel *in vivo* data on the behaviour of LPL protein concentration and activity under basal conditions and after i.v. heparin injection. Serum LPL protein concentrations were similar in 42 CLL patients compared to 14 non-CLL controls and 3 HI. This was also true for high and low LPL mRNA expressing patients. The functional post-heparin test leads to an increase of LPL protein concentration in serum and activity in plasma in normal subjects. Surprisingly, both LPL serum levels and enzymatic activities in CLL patients were comparable to those found in healthy individuals according to data reported in the literature [32,33]. Considering the higher lymphocyte counts in CLL patients compared to healthy individuals, and the fact that LPL protein has been demonstrated in CLL cells [12], one would have expected a much higher increase in LPL protein concentration, in high LPL expressing cases. Thus, our findings could indicate that (i) part of the LPL is retained by an alternative mechanism on the surface of CLL cells leaving it non-responsive to heparin treatment, (ii) that the amount of surface LPL on CLL cells is much less than previously thought, and/or (iii) LPL in CLL exists mostly as non-active monomer. Alternatively LPL might be bound by lymphocytes similarly as described for endothelial cells [28] yet their release might be blocked. Our *in vitro* data support this hypothesis since (i) post-heparin LPL levels resembled

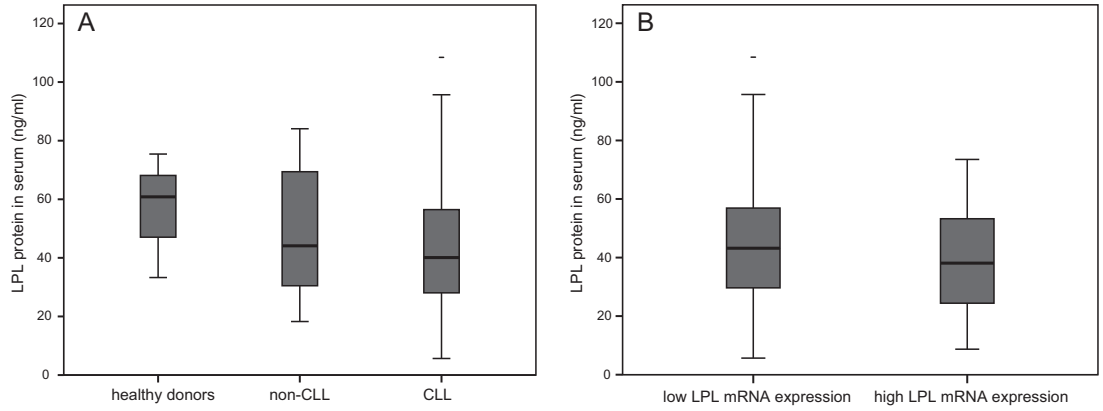

Fig. 1. (A) Box plot showing LPL protein concentrations in the sera of CLL patients ($N = 42$) *vs.* non-CLL patients (lymphoma patients in complete remission; $N = 14$) *vs.* healthy donors ($N = 3$). (B) Box plot showing LPL protein concentrations in the sera of CLL patients ($N = 42$) according to high and low mRNA expression ($N = 16$ in high expressing, $N = 26$ in low expressing group).

Table 1
Median LPL protein serum concentration (ng/ml) and plasma activity (μM/ml/h) upon heparin treatment *in vivo*. Data listed correspond to before (0), 10 and 20 min after heparin injection. Normal range according to literature: LPL protein mass 40.3 ± 14.42 ng/ml [34] and 58.3 ± 43.5 ng/ml [33] pre-heparin; 334.1 ± 71.5 ng/ml [34] and 323 ± 40.6 ng/ml [33] post-heparin. Post-heparin LPL activity range of 3.33–24.41 μM/ml/h in normal subjects. [30].

	LPL protein 0 (ng/ml)	LPL protein 10 (ng/ml)	LPL protein 20 (ng/ml)
CLL patients with high LPL mRNA expression ($N = 5$)	16.11	214.33	332.78
CLL patients with low LPL mRNA expression ($N = 8$)	13.08	219.68	386.65
	LPL activity 0 (μM/ml/h)	LPL activity 10 (μM/ml/h)	LPL activity 20 (μM/ml/h)
CLL patients with high LPL mRNA expression ($N = 5$)	7.25	15.52	19.13
CLL patients with low LPL mRNA expression ($N = 8$)	7.45	20.01	20.57

those of normal individuals and (ii) heparin incubation of CLL cells *ex vivo* did not result in elevated LPL levels in the supernatant under serum-free conditions.

Our observations complement data published by Mansouri et al. [24] who showed that LPL protein expression follow LPL mRNA expression in CLL subgroups defined by IGHV mutational status. Using cell lysates, these authors found extremely low levels of LPL activity. To determine specific LPL activity in mutated and unmutated cases, they used the ratio of LPL activity per amount of LPL protein expressed. LPL in mutated cases showed statistically significant higher specific activity compared to unmutated cases. This would indicate that, while having lower mRNA and protein expression, LPL in mutated CLL cases is more active compared to unmutated cases. Based on the low levels of LPL activity in total, however, the authors concluded that a function of catalytically active LPL in CLL might be questionable. Such a low enzyme activity would fit well with the reported reduced HDL-C levels in leukaemia patients [27].

Taken together, these data suggest that the general impact of high LPL expression in high-risk CLL cells on lipoprotein metabolism might be low due to the lack of active secretion of LPL. Noteworthy in this respect is the absence of noticeable effects in lipid metabolism in high-risk CLL patients. This could point to a possible function as a surface receptor or bound bridging molecule [28,39]. In addition, an intracellular function may be operative as high LPL mRNA expression is associated with a high cytoplasmic protein expression [12].

If LPL plays a role in energy supply for CLL cells one could expect better survival of tumour cells when supplied with LPL under cell culture conditions. However, when incubating primary CLL cells with purified LPL protein *in vitro* with and without FCS, we could not observe any changes in cell viability and LPL mRNA expression in high and low expressing CLL cases *vs.* negative controls. Since CLL cells are known to enter apoptosis relatively soon when cultured without feeder cells, one would have expected a survival advantage when supplemented with LPL. Our results might indicate that LPL

Table 2
List of genes which were co-regulated with LPL knock down in microarray analysis ($N = 10$) and subsequently selected for validation. The last two columns show knock down efficiencies in validation experiments in an additional set of 7 samples detected by real time PCR. Negative values represent downregulation after LPL knock down.

Gene symbol	Knock down-microarray analysis ($N = 10$)		Knock down-validation and real time PCR ($N = 7$)	
	Downregulation in *N* samples	Median relative downregulation (%)	Downregulation in *N* samples	Median relative downregulation (%)
LMAN1[a]	8	−34	6	−29
RANBP2[b]	8	−53	4	−39
SSB[c]	8	−41	4	−23
STXBP3[d]	8	−28	6	−18
LARP7[e]	8	−26	5	−17

[a] LMAN1 (lectin, mannose-binding 1): type 1 integral membrane protein, mannose-specific lectin.
[b] RANBP2 (RAN-binding protein 2): GTP-binding protein of the RAS superfamily, localized in the nuclear membrane, involved in nuclear-cytoplasmic transport of proteins.
[c] SSB (Sjogren syndrome antigen B): RNA-binding protein, involved in various aspects of RNA metabolism.
[d] STXBP3 (syntaxin-binding protein 3): involved in fatty acid induced insulin resistance in skeletal muscle cells.
[e] LARP7 (La ribonucleoprotein domain family, member 7): RNA-binding protein and transcription factor.

protein supplemented in the culture medium is not taken up by the cells or, if so, does not influence cell survival. Likewise, cell survival, both *in vivo* and *in vitro*, and LPL mRNA expression were unaffected by heparin treatment.

In this context it is noteworthy that Pallasch et al. compared LPL activity between CLL cells and B-cells of healthy individuals and found 4.5-fold higher activity in CLL in cell lysates, which include, in contrast to our study, both cell surface and intracellular LPL [25]. They did not compare LPL activity between high and low LPL expressing CLL patients. Treating cells with Orlistat, they observed a decrease of LPL activity in CLL lysates and an increase in apoptosis compared to healthy B-cells and PBMC. However, the authors did not find LPL specific susceptibility to Orlistat in CLL since the effect of this lipase inhibitor was similar in LPL negative and LPL positive sample groups. In addition, this work group evaluated the influence of erufosine, which also targets lipid metabolism, and found higher induction of apoptosis in CLL cells compared to healthy PBMC [26].

In order to further address a possible functional role of LPL for lymphocyte survival in CLL, we have performed a specific knock down of LPL by siRNA. With all limitations of this technique in CLL cells, which are difficult to transfect, we could not observe an increased cell death within 48 h. Nevertheless, a small number of genes appeared to be coregulated with LPL, in particular STXBP3. Interestingly, STXBP3 has been shown to be involved in fatty acid induced insulin resistance in skeletal muscle cells. Also, with human LPL stably transfected C2C12 myoblast cells had increased levels of STXBP3 mRNA and protein [35]. These data, together with our results, indicate that this gene behaves as a downstream target of LPL. LMAN1, a type 1 integral membrane protein, is a mannose-specific lectin. It stimulates IgM polymerization and mutations in the gene are associated with coagulation defects [40]. LARP7 is a RNA-binding protein and transcription factor. In gastric cancer, LARP7 was found to function as tumour suppressor gene [41]. SSB (LARP3), like LARP7 a RNA-binding protein, also is involved in various aspects of RNA metabolism. RANBP2, a GTP-binding protein of the RAS superfamily, is localized in the nuclear membrane being involved particularly in nuclear-cytoplasmic transport of proteins. In addition, it has been reported to be fused to ALK in atypical myeloproliferative leukaemia in children resulting in a RANBP2-ALK fusion transcript [42]. This low number of LPL co-regulated genes is due to the biological heterogeneity among CLL samples, the known difficulties, when applying knock down techniques to primary CLL cells, and to the hence necessarily very restrictive bioinformatic analysis.

Lipoprotein lipase mRNA expression is an excellent prognostic marker in CLL. What causes LPL overexpression, however, is not clear and studies are complicated by the fact that the regulation of this gene is tissue specific at transcriptional, posttranscriptional and posttranslational levels [43]. In this respect, a study reported only recently that external stimuli induced demethylation of the R1 region of LPL leading subsequently to an upregulation of LPL expression both on mRNA and protein level [44]. The functional consequences of LPL mRNA overexpression, however, are unclear. Alterations in lipid metabolism as reported for a number of malignancies [36–38] might suggest an involvement in tumour energy supply and, potentially, survival advantage. All our experiments, in contrast, fail to show such a role for LPL at least in short-term experiments.

Further studies will be necessary to uncover a role of LPL in CLL, in particular, long term co-culture observations of CLL cells with stable LPL knock down are warranted. Furthermore, it may not be ruled out that LPL overexpression is the consequence of general cellular stress in the tumour cells and therefore a lack of functional significance in CLL may not be excluded.

Conflict of interest statement

The authors disclose no conflicts of interest.

Acknowledgements

The excellent technical assistance of Susanne Schnabl, Martin Hilgarth, and Markus Jeitler is appreciated. This work was supported by the Austrian Federal Ministry of Science through the Gen-AU-Child project (GZ 200.136/1 – VI/1/2005) and the Austrian Society of Hematology and Oncology (OEGHO).

Authors' contributions. E.P. performed research, analyzed data and wrote the paper. S.T. and M.B. carried out gene expression arrays and performed bioinformatics analysis. G.K. measured LPL protein and enzyme activity levels. M.G. performed research. S.E., D.H., K.F. and C.S. collected and analyzed data. T.L. performed research. M.S. designed study and analyzed data. U.J. and K.V. designed the study, analyzed the data and wrote the paper.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.leukres.2013.02.008.

References

[1] Van Bockstaele F, Verhasselt B, Philippe J. Prognostic markers in chronic lymphocytic leukemia: a comprehensive review. Blood Rev 2009;23(1):25–47.
[2] Redaelli A, Laskin BL, Stephens JM, Botteman MF, Pashos CL. The clinical and epidemiological burden of chronic lymphocytic leukaemia. Eur J Cancer Care (Engl) 2004;13(3):279–87.
[3] Hamblin TJ, Orchard JA, Ibbotson RE, Davis Z, Thomas PW, Stevenson FK, et al. CD38 expression and immunoglobulin variable region mutations are independent prognostic variables in chronic lymphocytic leukemia, but CD38 expression may vary during the course of the disease. Blood 2002;99(3):1023–9.
[4] Oscier DG, Gardiner AC, Mould SJ, Glide S, Davis ZA, Ibbotson RE, et al. Multivariate analysis of prognostic factors in CLL: clinical stage. IGVH gene mutational status, and loss or mutation of the p53 gene are independent prognostic factors. Blood 2002;100(4):1177–84.
[5] Meuleman N, Stamatopoulos B, Dejeneffe M, El Housni H, Lagneaux L, Bron D. Doubling time of soluble CD23: a powerful prognostic factor for newly diagnosed and untreated stage A chronic lymphocytic leukemia patients. Leukemia 2008;22(10):1882–90.
[6] Damle RN, Wasil T, Fais F, Ghiotto F, Valetto A, Allen SL, et al. Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. Blood 1999;94(6):1840–7.
[7] Kaderi MA, Kanduri M, Buhl AM, Sevov M, Cahill N, Gunnarsson R, et al. LPL is the strongest prognostic factor in a comparative analysis of RNA-based markers in early chronic lymphocytic leukemia. Haematologica 2011;96(8):1153–60.
[8] Wiestner A, Rosenwald A, Barry TS, Wright G, Davis RE, Henrickson SE, et al. ZAP-70 expression identifies a chronic lymphocytic leukemia subtype with unmutated immunoglobulin genes, inferior clinical outcome, and distinct gene expression profile. Blood 2003;101(12):4944–51.
[9] Haslinger C, Schweifer N, Stilgenbauer S, Dohner H, Lichter P, Kraut N, et al. Microarray gene expression profiling of B-cell chronic lymphocytic leukemia subgroups defined by genomic aberrations and VH mutation status. J Clin Oncol 2004;22(19):3937–49.
[10] Vasconcelos Y, De Vos J, Vallat L, Reme T, Lalanne AI, Wanherdrick K, et al. Gene expression profiling of chronic lymphocytic leukemia can discriminate cases with stable disease and mutated Ig genes from those with progressive disease and unmutated Ig genes. Leukemia 2005;19(11):2002–5.
[11] Trojani A, Di Camillo B, Tedeschi A, Lodola M, Montesano S, Ricci F, et al. Gene expression profiling identifies ARSD as a new marker of disease progression and the sphingolipid metabolism as a potential novel metabolism in chronic lymphocytic leukemia. Cancer Biomark 2011;11(1):15–28.
[12] Heintel D, Kienle D, Shehata M, Krober A, Kroemer E, Schwarzinger I, et al. High expression of lipoprotein lipase in poor risk B-cell chronic lymphocytic leukemia. Leukemia 2005;19(7):1216–23.
[13] Maloum K, Settegrana C, Chapiro E, Cazin B, Lepretre S, Delmer A, et al. IGHV gene mutational status and LPL/ADAM29 gene expression as clinical outcome predictors in CLL patients in remission following treatment with oral fludarabine plus cyclophosphamide. Ann Hematol 2009;88(12):1215–21.
[14] Nikitin EA, Malakho SG, Biderman BV, Baranova AV, Lorie YY, Shevelev AY, et al. Expression level of lipoprotein lipase and dystrophin genes predict survival in B-cell chronic lymphocytic leukemia. Leuk Lymphoma 2007;48(5):912–22.

[15] Van Bockstaele F, Pede V, Janssens A, Callewaert F, Offner F, Verhasselt B, et al. Lipoprotein lipase mRNA expression in whole blood is a prognostic marker in B cell chronic lymphocytic leukemia. Clin Chem 2007;53(2):204–12.
[16] van't Veer MB, Brooijmans AM, Langerak AW, Verhaaf B, Goudswaard CS, Graveland WJ, et al. The predictive value of lipoprotein lipase for survival in chronic lymphocytic leukemia. Haematologica 2006;91(1):56–63.
[17] Nuckel H, Huttmann A, Klein-Hitpass L, Schroers R, Fuhrer A, Sellmann L, et al. Lipoprotein lipase expression is a novel prognostic factor in B-cell chronic lymphocytic leukemia. Leuk Lymphoma 2006;47(6):1053–61.
[18] Oppezzo P, Vasconcelos Y, Settegrana C, Jeannel D, Vuillier F, Legarff-Tavernier M, et al. The LPL/ADAM29 expression ratio is a novel prognosis indicator in chronic lymphocytic leukemia. Blood 2005;106(2):650–7.
[19] Antic D, Mihaljevic B, Cokic V, Fekete MD, Djurasevic TK, Pavlovic S, et al. Patients with early stage chronic lymphocytic leukemia: new risk stratification based on molecular profiling. Leuk Lymphoma 2011;52(7):1394–7.
[20] Stamatopoulos B, Meuleman N, Haibe-Kains B, Saussoy P, Van Den Neste E, Michaux L, et al. microRNA-29c and microRNA-223 down-regulation has in vivo significance in chronic lymphocytic leukemia and improves disease risk stratification. Blood 2009;113(21):5237–45.
[21] Stamatopoulos B, Meuleman N, De Bruyn C, Pieters K, Anthoine G, Mineur P, et al. A molecular score by quantitative PCR as a new prognostic tool at diagnosis for chronic lymphocytic leukemia patients. PLoS ONE 2010;5(9).
[22] Kotaskova J, Tichy B, Trbusek M, Francova HS, Kabathova J, Malcikova J, et al. High expression of lymphocyte-activation gene 3 (LAG3) in chronic lymphocytic leukemia cells is associated with unmutated immunoglobulin variable heavy chain region (IGHV) gene and reduced treatment-free survival. J Mol Diagn 2010;12(3):328–34.
[23] Bilban M, Heintel D, Scharl T, Woelfel T, Auer MM, Porpaczy E, et al. Deregulated expression of fat and muscle genes in B-cell chronic lymphocytic leukemia with high lipoprotein lipase expression. Leukemia 2006;20(6):1080–8.
[24] Mansouri M, Sevov M, Fahlgren E, Tobin G, Jondal M, Osorio L, et al. Lipoprotein lipase is differentially expressed in prognostic subsets of chronic lymphocytic leukemia but displays invariably low catalytical activity. Leuk Res 2010;34(3):301–6.
[25] Pallasch CP, Schwamb J, Konigs S, Schulz A, Debey S, Kofler D, et al. Targeting lipid metabolism by the lipoprotein lipase inhibitor orlistat results in apoptosis of B-cell chronic lymphocytic leukemia cells. Leukemia 2008;22(3):585–92.
[26] Konigs SK, Pallasch CP, Lindner LH, Schwamb J, Schulz A, Brinker R, et al. Erufosine, a novel alkylphosphocholine, induces apoptosis in CLL through a caspase-dependent pathway. Leuk Res 2010;34(8):1064–9.
[27] Mulas MF, Abete C, Pulisci D, Pani A, Massidda B, Dessi S, et al. Cholesterol esters as growth regulators of lymphocytic leukaemia cells. Cell Prolif 2011;44(4):360–71.
[28] Mead JR, Irvine SA, Ramji DP. Lipoprotein lipase: structure, function, regulation, and role in disease. J Mol Med (Berl) 2002;80(12):753–69.
[29] Wong H, Schotz MC. The lipase gene family. J Lipid Res 2002;43(7):993–9.
[30] Henderson AD, Richmond W, Elkeles RS. Hepatic and lipoprotein lipases selectively assayed in postheparin plasma. Clin Chem 1993;39(2):218–23.
[31] Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP. Summaries of Affymetrix GeneChip probe level data. Nucleic Acids Res 2003;31(4):pe15.
[32] Holzl B, Kraft HG, Wiebusch H, Sandhofer A, Patsch J, Sandhofer F, et al. Two novel mutations in the lipoprotein lipase gene in a family with marked hypertriglyceridemia in heterozygous carriers. Potential interaction with the polymorphic marker D1S104 on chromosome 1q21-q23. J Lipid Res 2000;41(5):734–41.
[33] van Beek AP, van Barlingen HH, de Ruijter-Heijstek FC, Jansen H, Erkelens DW, Dallinga-Thie GM, et al. Preferential clearance of apoB-48-containing lipoproteins after heparin-induced lipolysis is modulated by lipoprotein lipase activity. J Lipid Res 1998;39(2):322–32.
[34] Wicher I, Sattler W, Ibovnik A, Kostner GM, Zechner R, Malle E. Quantification of lipoprotein lipase (LPL) by dissociation-enhanced lanthanide fluorescence immunoassay. Comparison of immunoreactivity of LPL mass and enzyme activity of LPL. J Immunol Methods 1996;192(1/2):1–11.
[35] Schlaepfer IR, Pulawa LK, Ferreira LD, James DE, Capell WH, Eckel RH. Increased expression of the SNARE accessory protein Munc18c in lipid-mediated insulin resistance. J Lipid Res 2003;44(6):1174–81.
[36] Santos CR, Schulze A. Lipid metabolism in cancer. FEBS J 2012;279(15):2610–23.
[37] Bhatt AP, Jacobs SR, Freemerman AJ, Makowski L, Rathmell JC, Dittmer DP, et al. Dysregulation of fatty acid synthesis and glycolysis in non-Hodgkin lymphoma. Proc Natl Acad Sci U S A 2012;109(29):11818–23.
[38] Tirado-Velez JM, Joumady I, Saez-Benito A, Cozar-Castellano I, Perdomo G. Inhibition of fatty acid metabolism reduces human myeloma cells proliferation. PLoS ONE 2012;7(9):pe46484.
[39] Stein Y, Stein O. Lipoprotein lipase and atherosclerosis. Atherosclerosis 2003;170(1):1–9.
[40] Anelli T, Ceppi S, Bergamelli L, Cortini M, Masciarelli S, Valetti C, et al. Sequential steps and checkpoints in the early exocytic compartment during secretory IgM biogenesis. EMBO J 2007;26(19):4177–88.
[41] Cheng Y, Jin Z, Agarwal R, Ma K, Yang J, Ibrahim S, et al. LARP7 is a potential tumor suppressor gene in gastric cancer. Lab Invest 2012;92(7):1013–9.
[42] Rottgers S, Gombert M, Teigler-Schlegel A, Busch K, Gamerdinger U, Slany R, et al. ALK fusion genes in children with atypical myeloproliferative leukemia. Leukemia 2010;24(6):1197–200.
[43] Wang H, Eckel RH. Lipoprotein lipase: from gene to obesity. Am J Physiol Endocrinol Metab 2009;297(2):E271–88.
[44] Moreno P, Abreu C, Borge M, Morande P, Pegazzano M, Palacios F, et al. Lipoprotein lipase expression in unmutated CLL patients is the consequence of a demethylation process induced by the microenvironment. Leukemia 2012, http://dx.doi.org/10.1038/leu.2012.212.